RESEARCH LETTER

Mycoplasma gallisepticum as the first analyzed bacterium in which RNA is not polyadenylated

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Abstract

The addition of poly(A)-tails to RNA is a phenomenon common to almost all organisms. In addition to most eukaryotic mRNAs possessing a stable poly(A)-tail, RNA is polyadenylated as part of a degradation mechanism in prokaryotes, organelles, and the eukaryotic nucleus. To date, only very few systems have been described wherein RNA is metabolized without polyadenylation, including several archaea and yeast mitochondria. The minimal genome of the parasitic bacteria, *Mycoplasma*, does not encode homologs of any known polyadenylating enzyme. Here, we analyze polyadenylation in *Mycoplasma gallisepticum*. Our results suggest this organism as being the first described bacterium in which RNA is not polyadenylated.

Introduction

Polyadenylation is a general posttranscriptional process characterizing almost all living organisms (Shatkin & Manley, 2000; de Moor & Richter, 2001; Edmonds, 2002; Mangus *et al*., 2003). Generally, polyadenylation can be classified into two groups. The first group consists of stable poly(A)-tails located at the 3′ end of most eukaryotic mRNAs, as well as animal and trypanosome mitochondrial transcripts. The second group contains unstable poly(A)-tails that are considered to be part of the polyadenylation-assisted degradation pathway present in bacteria, some archaea, organelles, and the nucleus of eukaryotic cells (Dreyfus & Regnier, 2002; Kushner, 2004; Houseley *et al*., 2006; Slomovic *et al*., 2006a, b; West *et al*., 2006; Vanacova & Stefl, 2007). A different classification of polyadenylation could be made according to the nature of the tails. One group of poly(A)-tails is characterized by homopolymeric tails composed of only adenosines. This group includes all of the stable tails of eukaryotic mRNAs, the stable and unstable tails of animal mitochondria, the chloroplasts of *Arabidopsis*, and many bacteria, such as *Escherichia coli*. The second group consists of heteropolymeric tails containing all four nucleotides. These heteropolymeric poly(A)-rich tails were found in bacteria, such as *E. coli*, *Bacillus subtilis*, *Cyanobacteria*, hyperthermophilic and several methanogenic archaea, spinach chloroplast, and human rRNA (Lisitsky *et al*., 1996; Rott *et al*., 2003; Mohanty *et al*., 2004; Campos-Guillen *et al*., 2005; Portnoy *et al*., 2005; Portnoy & Schuster, 2006; Slomovic *et al*., 2006a, b). While the homopolymeric tails are synthesized by poly(A)-polymerases, which, in bacteria, belong to the nucleotidyltransferase (Ntr) super family, the heteropolymeric tails are produced by the phosphorolytic enzymes polynucleotide phosphorylase (PNPase) and the archaeal exosome (Mohanty & Kushner, 2000; Yehudai-Resheff *et al*., 2001; Portnoy *et al*., 2005).

Within the framework of our studies on polyadenylation in different organisms, we examined the question of whether there are organisms that metabolize RNA without polyadenylation. To this end, sequenced genomes in the genomic data bank were searched for those lacking any known PAP, PNPase, and archaeal exosome homologs. Halophilic and several methanogenic archaea were identified and analyzed as the first organisms where no polyadenylation takes place (Portnoy *et al*., 2005; Portnoy & Schuster, 2006). In these organisms, the polyadenylation-independent degradation of the RNA pathway is derived by the hydrolytic exoribonuclease RNAse R (Portnoy & Schuster, 2006). Interestingly, in hyperthermophilic and other methanogenic archaea where polyadenylation does take place, the tails are

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heteropolymeric and the polymerase is the archaeal exosome, which is very similar to PNPase (Lorentzen *et al.*, 2005; Portnoy *et al.*, 2005; Buttner *et al.*, 2006; Portnoy & Schuster, 2006). A similar situation occurs in yeast mitochondria, where no PAP or PNPase is present, and accordingly, no polyadenylation exists (Dziembowski *et al.*, 2003; Schafer *et al.*, 2005). The degradation is carried out by a protein complex (mtEXO) composed of an RNAse R homolog (Diss1p) and an RNA helicase (Suv3p) (Dziembowski *et al.*, 2003; Dziembowski *et al.*, 2007).

The parasitic bacteria of the genus *Mycoplasma* are characterized by the lack of a cell wall and the existence of a small genome containing a minimal gene set. The homolog genes encoding PNPase, Ntr, and PAP I are missing. Moreover, an RNAse R homolog is present in the genome, which is essential for viability, and was shown to be the single exoribonuclease that degrades RNA and processes the 3′ end of tRNAs (Lalonde *et al.*, 2007).

Here, we describe *Mycoplasma gallisepticum*, an avian parasite, as the first member of the bacteria domain in which polyadenylation does not take place. Therefore, *Mycoplasma* is added to the short list of organisms and an organelle where RNA is metabolized without any polyadenylation.

Materials and methods

Organism and RNA extraction

Mycoplasma gallisepticum were grown in the Division of Avian and Aquatic Diseases, Kimron Veterinary Institute, Bet-Dagan, Israel, as described (Lysnyansky *et al.*, 2005). *Synechocystis PCC 6803* and *Arabidopsis* were grown as described previously (Rott *et al.*, 2003). Purification of RNA was performed using the MasterPure^{TM} RNA Purification kit (Epicentre).

Determination of poly(A)-tails

RNA purified from *M. gallisepticum* (20 μg), *E. coli* (7 μg), *Halferax volcanii* (20 μg), or from *Arabidopsis thaliana* (5 μg) was 3′-end-labeled with [^{32}P]-pCp and T4 RNA ligase for 24 h at 4 °C before digestion with 25 μg of RNAse A and 300 units of RNAse T1 for 1 h at 37 °C (Portnoy *et al.*, 2005). Poly(A)-tails were resolved on a 14% denaturing polyacrylamide gel containing 7 M urea and detected by autoradiography.

Analysis of poly(A)-tails by oligo(dT)-primed reverse transcriptase (RT)-PCR and the Smart method

Oligo(dT)-primed cDNA was synthesized using StrataScript^{R} 5.0 Multi-temperature Reverse Transcriptase (Stratagene) and oligo $(dT)_{10}$-Adaptor for 2 h at 48 °C. The cDNA was PCR amplified using adaptor oligo and one of the gene-specific primers listed in supplementary Table S1. The resulting PCR products were cloned to the pGEM-T Easy vector and sequenced (Portnoy *et al.*, 2005).

The Smart RT-PCR technique was performed according to the manufacturer's protocol (Clontech). The amplified products were ligated to the pGEM-T Easy vector and sequenced.

Results

No genes encoding polyadenylation enzymes are present in the *Mycoplasma* genome

We screened a variety of *Mycoplasma* sequenced genomes from the NCBI database for gene-encoding homologs of known poly(A) polymerases. Any putative PAP, Ntr, or PNPase could be detected (Table 1). In addition, gene homologs to RNAse PH or archaea exosome proteins were not present (Table 1). In this respect, *Mycoplasma* is similar to halophilic archaea, the first class of organisms described to metabolize RNA without polyadenylation (Portnoy *et al.*, 2005). Among the RNA metabolism enzymes, the small *Mycoplasma* genome contained endoribonuclease RNAse J and exoribonuclease RNAse R (Table 1 and supplementary Table S2).

No polyadenylated RNA could be observed in *Mycoplasma*

Because *Mycoplasma*, similar to halophilic archaea, does not contain a gene encoding one of the known polyadenylating enzymes, we hypothesized that no polyadenylation occurs. In order to verify this hypothesis experimentally, RNA was purified from *M. gallisepticum*, a mycoplasmal pathogen of

Table 1. RNA metabolism enzymes present in *Mycoplasma*

Protein	Presence in *Mycoplasma* genomes
RNAse J (endoribonuclease)	+
RNAse E (endoribonuclease)	−
RNAse PH (exonuclease, phosphorylase)	−
PNPase (exonuclease, phosphorylase)	−
RNAse II/R (exonuclease, hydrolase)	+
tRNA Ntrs (CCA transferase)	−
Ntrs type poly(A)-polymerase	−

The genomic BLAST program (http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi) was used to search the *Mycoplasma* genomes in the NCBI database for the protein homologs listed above. Plus and minus signs indicate the presence or absence of the corresponding gene homologs. The RNAse J and RNAse R homologs of *Mycoplasma* are listed in supplementary Table S2.

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poultry, and from halophilic archaea and *Arabidopsis* as negative and positive controls, respectively, and checked for inactiveness (Fig. 1a). In addition, *E. coli* RNA served as a control for the detection of unstable poly(A)-tails. RNA was then labeled with [^{32}P]-pCp at the 3′ end, and digested with RNAse A and RNAse T1. Both these enzymes digest following G, C, and T, and therefore only poly(A)-tails located at the 3′ end of the RNA were detected (Portnoy *et al*., 2005). The results showed no signals related to poly(A)-tails (Fig. 1b). This was also the case for the halophilic archaeon *H. volcanii* where, as described previously, no polyadenylation occurs (Portnoy *et al*., 2005). However, short poly(A)-tails were detected when analyzing RNA of *E. coli* (Fig. 1b). In addition, long poly(A)-tails were detected on analyzing the RNA of the higher plant *A. thaliana*, where most mRNAs 3′ ends contain stable poly(A)-tails (Fig. 1b). It should be noted that as most mRNAs in *Arabidopsis* are decorated with a stable poly(A)-tail, a smaller amount of this RNA relatively to the *Mycoplasma* and Archaea was analyzed in this experiment. These results suggested that, as predicted from the genomic analysis, RNA is metabolized in *Mycoplasma* without polyadenylation. In order to verify that indeed no

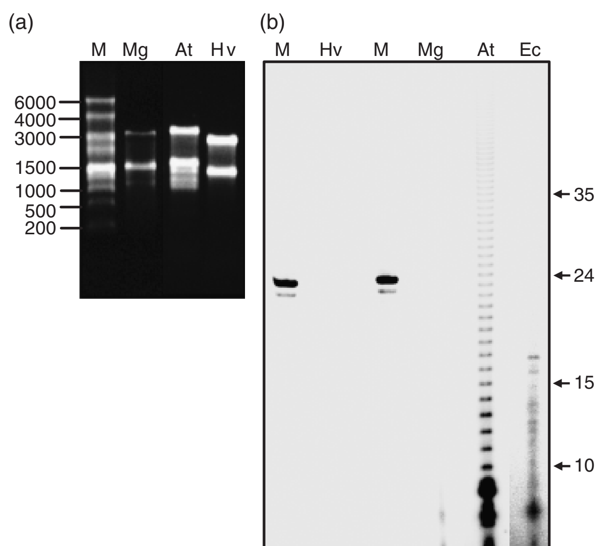

Fig. 1. No poly(A)-tails are present in *Mycoplasma gallisepticum*. (a) Total RNA was purified from *Mycoplasma gallisepticum* (Mg), the higher plant *Arabidopsis thaliana* (At), the halophilic archaea *Haloferax volcanii* (Hv), and *Escherichia coli* (Ec). In order to verify that the purified RNA remained intact, it was resolved in 1% agarose gel and stained with EtBr. RNA size markers, as shown to the left in nucleotide (nt) length, were fractionated in the first lane (M). (b) Purified RNA (20 μg of *H. volcanii*, 7 μg of *E. coli*, 20 μg of *M. gallisepticum*, and 5 μg from Arabidopsis) was [^{32}P]-labeled at the 3′ end and digested to completion with RNAse T1 and RNAse A, following fractionation on 14% denaturing polyacrylamide gel electrophoresis. A [^{32}P]-labelled 24 nt oligonucleotide was fractionated on the same gel as a size marker (M). The length of the tails in nucleotides is shown on the right.

polyadenylation exists, attempts to observe putative poly(A)-tails were carried out using two RT-PCR methods.

Analysis of polyadenylation using RT-PCR methods confirmed that no poly(A)-tails are present in *Mycoplasma*

In order to confirm the results that no polyadenylation occurs in *Mycoplasma*, we applied two additional sensitive methods based on oligo(dT)-primed cDNA synthesis and PCR amplification. In the first method, the oligo(dT)-primed cDNA is PCR amplified using gene-specific and adaptor primers (supplementary Table S1). This method has been used by many laboratories to detect nonabundant poly(A)-tails decorating truncated RNAs in bacteria, archaea, chloroplast, and mitochondria, as well as nucleus-encoded RNAs. As a positive control, RNA isolated from the cyanobacteria *Synechocystis* PCC 6803 was applied. The RNA of this organism was characterized previously to contain nonabundant and heteropolymeric tails produced by the enzyme PNPase (Rott *et al*., 2003). Because the PCR amplification is so powerful, we knew from our work with halophilic archaea and yeast mitochondria that amplified products are observed even when no polyadenylation occurs (Portnoy *et al*., 2005). Therefore, the RT-PCR amplification products observed were cloned and analyzed by DNA sequencing.

Under these conditions and when no polyadenylated transcripts are present, RT-PCR products could be obtained as a result of annealing of the oligo(dT_{10}) primer to a stretch of several adenosines located in the mRNA sequence. Usually, a stretch of four to five adenosines could initiate the reverse transcription reaction. Another artifact could be obtained as a result of miss-priming of the gene-specific or the adaptor primers to RNA or the residual genomic DNA that remained in the reaction mixture (Table S3 in the supplementary material). Therefore, only RT-PCR products containing the gene-specific primer, followed by the transcript sequence and then a poly(A), or poly(A)-rich tails were counted. When a stretch of several adenosins was located in the transcript at the place of the poly(A)-tail, only tails containing more than 10 adenosines (the number of adenosins in the reverse transcription primer) were counted. Lastly, when a heteropolymeric tail is identified, its nucleotide sequence is verified not to be present in the general databank of all nucleotide sequences in order to verify that no contamination of the RT-PCR procedure was obtained (supplementary Tables S3 and S4).

The results revealed that out of 38 analyzed sequences obtained using *Mycoplasma* RNA and specific primers to the 16S rRNA gene, $tRNA^{asp}$, and *VacB*, none was found to harbor a poly(A)-tail (Fig. 2, Table 2 and supplementary Table S3). The rRNA and tRNA transcripts were chosen for

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this analysis because of their relatively high abundance in the cell. For comparison, out of the 21 *Synechocystis* sequences analyzed as a positive control, 15 were found to be decorated with heteropolymeric tails, as described previously (Fig. 2, Table 2 and supplementary Table S4) (Rott *et al*., 2003). Together, these results confirmed the suggestion that no polyadenylation occurs in *Mycoplasma*.

The oligo(dT)-primed RT-PCR technique is very powerful in the detection of nonabundant and minute amounts of polyadenylated transcripts. However, it is limited to those transcripts for which specific primers for PCR amplification are used (Fig. 2). In order to verify that there is no unknown transcript that is specifically polyadenylated in *Mycoplasma*, we used the more general method of oligo(dT) primed Smart RT-PCR analysis. In this technique, there is no use of gene-specific primers, and therefore, any polyadenylated transcripts, if present, would be detected. Out of the 13 amplification products obtained using *Mycoplasma* RNA as a substrate and analyzed by DNA sequencing, none contained a poly(A)-tail (Table 2, Fig. 3 and supplementary Table S5). For comparison, six out of the nine clones were found to be decorated with heteropolymeric tails in *Synechocystis*, used here as a positive control (Table 2 and Fig. 3). Together, global analysis of 3′ pCp-labeled RNAs and oligo(dT) RT-PCR amplified extremities supports the hypothesis that no polyadenylation, either of heteropolymeric or homopolymeric tails, takes place in *M. gallisepticum*.

Fig. 2. No polyadenylation could be detected in *Mycoplasma gallisepticum* by the oligo(dT)-gene-specific primer RT-PCR method. Oligo(dT)-primed RT-PCR analysis was performed on RNA preparations of *M. gallisepticum* (a) or the cyanobacteria *Synechocystis* sp. PCC 6803 (b). The gene-specific primers used for PCR amplification are indicated arrows. The positions of the truncated RNAs as located from the sequence of the amplified cDNAs are shown as horizontal lines. Poly(A)-tails added to the truncated RNA, only in the case of *Synechocystis* RNA, are marked with numbered vertical lines, and the sequence of the corresponding tail is presented below. The sequences of the *Mycoplasma* negative clones are shown in supplementary Tables S3 and S4.

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Discussion

Almost all organisms use posttranscriptional polyadenylation for RNA metabolism. The polyadenylation-assisted degradation pathway, where the addition of poly(A) or poly(A)-rich tails tags RNA molecules for rapid exonucleolytic degradation, was first analyzed in *E. coli* and then found in other bacteria, some archaea, organelles, and nucleus-encoded transcripts of eukaryotic cells (Shatkin & Manley, 2000; de Moor & Richter, 2001; Dreyfus & Regnier, 2002; Edmonds, 2002; Mangus *et al*., 2003; Kushner, 2004; Slomovic *et al*., 2005; Houseley *et al*., 2006; West *et al*., 2006; Vanacova & Stefl, 2007). In addition, stable poly(A)-tails are added to the 3′ ends of animals' and trypanosomes' mitochondria, as well as most eukaryotic mRNAs (Edmonds, 2002; Gagliardi *et al*., 2004). Only a few organisms and compartments were described that metabolize RNA without any polyadenylation, including halophilic and several methanogenic archaea, and the mitochondria of yeast (Dziembowski *et al*., 2003; Gagliardi *et al*., 2004; Portnoy *et al*., 2005; Schafer *et al*., 2005; Portnoy & Schuster, 2006). Indeed, these organisms lack known polyadenylating enzymes, such as PNPase, or the bacterial poly(A)-polymerase for bacteria and organelles, and the archaeal exosome for archaea. Because no homology to any known polyadenylating enzyme could be detected in the sequenced genomes of *Mycoplasma*, a small genome parasitic bacterium, we thought this might be the first bacterium described that metabolizes RNA without any polyadenylation. The results described in this work support this hypothesis.

Table 2. Summary of the oligo(dT)-Gene specific primers RT-PCR and Smart RT-PCR analysis

Method	Organism	Total clones analyzed	Number of clones with poly(A)
Oligo(dT)-RT-PCR	*Mycoplasma*	38	0
	Synechocystis	21	15
Smart-RT-PCR	*Mycoplasma*	13	0
	Synechocystis	9	6

How is RNA metabolized if no polyadenylation is present? In the polyadenylation-assisted degradation pathway, the poly(A)-tail is thought to provide an unstructured platform that attracts the exoribonuclease and enables this processive enzyme to overcome regions where structured RNA blocks or delays its activity. Our current view of the RNA degradation pathway in *E. coli* and organelles describes a concerted endo- and exonucleolytic event that, when required, polyadenylation-assists the exonucleolytic stage (Carpousis *et al*., 1999; Dreyfus & Regnier, 2002; Kushner, 2004; Slomovic *et al*., 2008). The observation that polyadenylation is generally observed in most organisms suggests that this polyadenylation assisted event is an important one and, hence, was preserved during evolution in most organisms, from bacteria to the nucleus of eukaryotes (Slomovic *et al*., 2008). In yeast mitochondria and archaea lacking polyadenylation and where no phosphorolytic activity is present, the RNAse R is responsible for exoribonuclease activity (Dziembowski *et al*., 2003; Portnoy & Schuster, 2006). The situation seems to be the same in *Mycoplasma*, where RNAse R is the only exoribonuclease found in the genome. This enzyme is essential since it effectively degrades structured molecules, and it was shown to be involved in several RNA processing reactions (Deutscher & Li, 2001; Cheng & Deutscher, 2005; Lalonde *et al*., 2007; Worrall & Luisi, 2007). Moreover, unlike the *E. coli* RNAse R and RNAse II enzymes, the *Mycoplasma genitalium* RNAse R was shown to display no specific poly(A) degradation activity, which supports the observation that, unlike the situation in *E. coli*, poly(A)-tails are not present in this organism (Lalonde *et al*., 2007).

In addition to the exoribonuclease RNAse R, the *Mycoplasma* genome contains a gene homologous to the recently described RNAse J (Table 1 and supplementary Table S2). This enzyme was first characterized in the bacteria *B. subtilis* as an endoribonuclease that, similar to RNAse E in *E. coli*, possibly performed the endonucleolytic cleavage in the degradation process (Even *et al*., 2005). This step is followed by exonucleolytic degradation or, alternatively, by polyadenylation of the cleavage product and subsequent exonucleolytic degradation (Dreyfus & Regnier, 2002; Slomovic *et al*.,

(a) *Mycoplasma*

No tails

(b) *Synechocystis*

1. $A_4G_2C_3A_8GCA_5G_2A_7GA_3GA_{10}GA_3GA_{10}$
2. $U_3AU_2G_2CAGAGA_3UAGAU_2A_5CA_7GACA_9$
3. $A_3C_2GA_2G_3AGCA_3G_3A_4G_2A_2GUA_{17}CA_3GA_7GA_9$
4. $AUGCUCA_3GA_2U_2A_4UA_2CA_3CAUCA_8GAUA_2CACA_2UA_5C_2AUA_4CA_3GA_9UA\ CA_2GAUA_{12}$
5. $U_2GU_2A_2CA_2GA_5GA_4GCGACG_2AGA_6CA_3GA_2CA_9$
6. $G_2CA_3GA_4G_2A_2GCA_5G_2A_6CG_7A_7UCA_4GA_2UA_9CA_2GACA_2GAUGA_3CA_2CACA_3UA_{17}UA_5$ $UCA_4CA_8GCA_4GAUA_9GACAG_2A_3GA_3GA_5CA_6CA_6GCA_2CAUA_9UA_2GA_2CA_2CAUA_5GA_5$ $GCAUA_6CA_6C_2A_2CA_3UCA_9$

Fig. 3. No polyadenylation could be detected in *Mycoplasma gallisepticum* using the Smart RT-PCR method. Smart RT-PCR analysis was performed on RNA preparations of *M. gallisepticum* (a) or the cyanobacteria *Synechocystis* sp. PCC 6803 (b). Heteropolymeric Poly(A)-tail were found only in the case of *Synechocystis* and the tails sequences are presented below. The sequences of the *Mycoplasma* negative clones are shown in supplementary Tables S3 and S5.

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2006). In *Mycoplasma*, the endonucleolytic cleavage by RNAse J could be followed by exonucleolytic digestion by RNAse R without polyadenylation. In addition, this enzyme was recently found to exhibit 5′–3′ exoribonuclease activity in *B. subtilis* in addition to the endonucleolytic one, making it the first bacterial enzyme described to work in this direction (Mathy *et al*., 2007). Therefore, the presence of 5′–3′ exonucleolytic degradation of RNA in *Mycoplasma* can also be predicted.

Although polyadenylation has been characterized in most organisms, the diversity of the pathways of RNA decay that emerged during evolution may explain the disappearance of this phenomenon in a small number of them. The evolutionary advantages, if any, of this change are not obvious. For example, the deletion of the two polyadenylating enzymes in *E. coli*, poly(A)-polymerase I and PNPase, results in lethality (Kushner, 2004). Similarly, deletion of the single polymerizing enzyme in *Synechocystis*, PNPase, is also lethal (Rott *et al*., 2003). Therefore, the polyadenylation assisted degradation pathway, when present, seems to be required for viability. Analysis of the molecular details of the polyadenylation-assisted and nonpolyadenylated degradation pathways in more organisms would help answer this question.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Oligonucleotides used as gene specific primers and for the oligo(dT)-primed and Smart RT-PCR analysis.

Table S2. Ribonucleases identified in *Mycoplasmas* genomes.

Table S3. The sequences of the dT-primed RT-PCR clones obtained from the analysis of *Mycoplasma*.

Table S4. *Synechocystis* 16S rRNA dT-RT-PCR clones.

Table S5. The clones obtained using the SMART protocol.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2008.01157.x (This link will take you to the article abstract.

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